POST-BOTTLING DEVELOPMENT OF SULFUR-LIKE OFF AROMAS IN FLAVORED WINES

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Christine Elizabeth Hansen

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ABSTRACT

Volatile sulfur-containing compounds are important contributors to wine aroma. These compounds have low sensory thresholds and can impact wine flavor even at low concentrations. Sulfur-like off aromas (SLOs) can develop during anaerobic wine storage. There are many reports in the literature of wine stored under low-oxygen conditions developing “rotten egg” and “reduced” aromas, but the appearance of this condition in flavored wines due to interactions between the wine and flavoring agent has not been previously reported. Sixteen flavoring agents were evaluated for their ability to form SLOs following their addition to wine and bottle storage. After three months, wines spiked with one of the flavoring agents exhibited an SLO described as “skunky/burnt rubber.” GC-Olfactometry was used to identify 3-methyl-2-buten-1-thiol (3-MBT) as the compound responsible for the “skunky/burnt rubber” aroma. 3-methylbut-2-en-1-ol, a likely precursor of 3-MBT, was quantified by GC-TOF-MS and determined to be significantly higher in the odorous flavor.

BIOGRAPHICAL SKETCH

Growing up in Minnesota, the author divided her free time between enjoying the warm winter weather and experimenting in the kitchen. She arrived at Cornell University in 2008 and quickly enrolled in the Enology and Viticulture program. During the summers, she gained hands-on experience as an intern at vineyards and wineries. Additionally, she loved working in the tasting room at two local breweries, where she got to talk with interesting people about beer all day. As an undergraduate student, she completed her honors thesis in Food Science working with Dr. Gavin Sacks on a project about the chemistry and sensory of flambé. Her interest in research led her to decide to stay in Ithaca to pursue a graduate degree in Food Science with a focus on wine aroma chemistry. She is excited about the opportunity to combine science with production to improve brewing and winemaking practices in the future.

To my parents, Steve and Laura.

To my sister, Kim.

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CHAPTER ONE

INTRODUCTION

Overview

Wine aroma is a result of many complex chemical and biochemical reactions. On a basic level, glucose and fructose present in grape juice are converted into ethanol and carbon dioxide through fermentation that, together with water, make up the majority of wine. Beyond these macro constituents, however, chemical compounds present in trace quantities can have a huge organoleptic impact on wine quality. Examples include esters, higher alcohols, vicinal diketones, and thiols. These aroma compounds can impact wine negatively and positively, depending on both species and concentration.

Aroma compounds typically associated with wine faults often have a rejection threshold close to their detection threshold. These compounds can form in the vineyard, at various stages in the winemaking process, and during storage. Microbial contamination by spoilage yeast and bacteria is a common source of off odors in wine. These microbes will produce compounds such as acetic acid, volatile phenols, and sulfur compounds. While inoculation by unwanted species can occur in the vineyard and in the winery, detrimental effects typically occur during the early stages of or just prior to fermentation [1-4]. A second common fault in wine is oxidation. Oxygen can be introduced during winemaking and during storage. Processes such as pumpovers and transfers can be a direct source for oxygen pick-up in winemaking, particularly if performed at low temperatures. During storage, oxygen in the bottle headspace can dissolve into the wine and ingress through certain types of closures. Oxidation can lead to a series of chemical changes in both red and white wines, including browning, loss of aromatics, and acetaldehyde production [5-7]. Faults that appear after bottling are particularly problematic because there is little that can

be done to correct the issue. Cork taint, caused by 2,4,6-trichloroanisole, is another example. This "musty, earthy" aroma is a result of microbial contamination and treating corks with chlorine or bleach solutions. It can occur in up to 5% of all wines [8].

Finally, volatile sulfur-containing compounds are responsible for a variety of off aromas in wine. Sulfur-like off aromas (SLOs) can form both during fermentation and after bottling. These compounds are the focus of this study and are discussed in detail throughout this chapter. The aim of this research is to better understand SLOs as a post-bottling wine fault, particularly in wines with added flavoring agents.

Sulfur Compounds in Wine

Sulfur-containing compounds are important flavor contributors to many foods including coffee, meat, beer and wine. Sulfur compounds can impart both negative and positive aromas in food, depending both on their concentrations and their structures. While there are many ways to group sulfur compounds in wine, the two categories discussed here are long-chain polyfunctional thiols and low-molecular weight thiols.

Long-chain polyfunctional thiols, such as 4-mercapto-4-methyl-pentan-2-one and 3-mercapto-1-hexanol, are some of the most potent aroma compounds in wine. Detection thresholds for these compounds range between .8 and 60 ng/L and are typically described as having cat urine and tropical fruit odors [9, 10]. While long-chain polyfunctional thiols are well-known contributors to the varietal character of Sauvignon blanc, they have also been found in Gewurtztraminer, Muscat, Riesling, Petit manseng and other *Vitis vinifera* varieties [11]. These compounds are present in wine grapes as precursors attached to an s-cysteine conjugate. During

fermentation, the thiol is cleaved from the precursor at the carbon-sulfur bond on the cysteine by ß-lyase from the yeast to become aroma active [12-14].

Low-molecular weight thiols, on the other hand, are typically associated with off aromas. These include sulfides, mercaptans, and thioacetates. Typical aroma descriptors of low-molecular weight thiols are cabbage, flatulence, egg, skunk, rubber, garlic, and onion [9]. These compounds also have low sensory thresholds. In red wine, the detection threshold for hydrogen sulfide (H_2S) is 1.1 µg/L and 1.8 µg/L for methyl mercaptan (CH_3SH) [9, 15]. Low-molecular weight thiols are generally derived from yeast during fermentation. While it has been shown that elemental sulfur in pesticides can be reduced to form H_2S, this rarely occurs except in cases of late application in the vineyard [16]. More commonly, H_2S is generated by yeast during fermentation via the sulfate reduction pathway (SRS), in which H_2S acts as an intermediate in sulfur-containing amino acid biosynthesis. If there is insufficient nutrient concentrations in the grape must, the SRS will be unable to synthesize amino acids due to lack of precursors but will continue to produce H_2S. This activity is highly variable by yeast strain; therefore certain types of yeast will produce more H_2S than others [17]. Off aromas due to volatile sulfur compounds can also develop during storage, this is discussed in detail in the following section.

Post-Bottling Development of Sulfur-like Off Aromas in Beverages

There are numerous reports of SLOs that appear during storage in wine and beer. It is important to gain a better understanding of this phenomenon because it is difficult to correct after the beverage is bottled. In beer, a notorious example is the “skunky” aroma that appears in light-struck beer and is caused by 3-methyl-2-buten-1-thiol (3-MBT). This compound is formed through the photolysis of iso-alpha acids in hops in the presence of sulfur-containing amino acids

and riboflavin, which acts as an oxidizer and photosensitizer [18-20]. While rare, this mechanism has also been reported in some white wines with high riboflavin content [21]. Packaging beverages in light protected bottles is the most common method used to prevent this off odor. A second SLO found in aged beer is "fresh onion" produced by dimethyl trisulfide (DMTS). The detection threshold for this compound is .1 μg/L. While it is present in trace levels in new beers, recent studies have shown that it can increase to suprathreshold levels during storage [10]. This also occurs in Japanese sake. Research by Isogai et al. reports that sake stored for 35 years at temperatures between 10-20°C has significantly more DMTS than unaged sake [22]. The formation of DMTS in beer and sake is likely due to oxidative reactions. A more recent study from the same group suggests that DMTS is generated from the Strecker degradation of methionine and can also be produced from other unidentified precursor compounds [23]. Finally, H_2S and dimethyl sulfide (DMS) have been shown to increase in beer during storage at higher temperatures (20°C-45°C) [24]. However, this phenomenon has not been well studied in beer, perhaps because beer is often consumed sooner after production than is wine.

There have been several reports of SLOs forming in wine stored in an anaerobic environment. In a study by Godden, et al. investigating fourteen closure types, wine stored under screw-cap in a low-oxygen environment produced SLOs after eighteen months [25] . Lopes et al. saw similar results with Sauvignon blanc in 2009: wine stored in low-oxygen conditions had increased concentrations of H_2S and "rotten egg" aromas [26]. The compounds responsible for post-bottling SLOs are likely H_2S and CH_3SH. Research by Rauhut et al. in 1998 found H_2S and CH_3SH in white commercials wines that showed evidence of off aromas. In a more recent study of sixty-eight commercial wines characterized as having SLOs, sixty-one contained H_2S and fifty-eight contained CH_3SH in concentrations well above sensory threshold [15]. In 2011,

Ugliano et al. demonstrated that Sauvignon blanc stored in an anaerobic environment had increased concentrations of H_2S and CH_3SH [27]. The same group reported the same phenomenon in Shiraz wine [28]. Thus, numerous studies have confirmed the prevalence of SLOs as a post-bottling wine fault.

Mechanisms for Sulfur-Like Off Aroma Formation in Wine

While post-bottling SLOs are a well-recognized wine fault, there is little agreement in the literature about its formation mechanism. One hypothesis proposes that disulfides are converted to mercaptans under reducing conditions. Bobet et al. first reported this in a study that investigated the conversion of diethyl disulfide (DEDS) to ethyl mercaptan (CH_3CH_2SH) in model wine. CH_3CH_2SH concentration increased after seven hundred days of storage [29]. Research by Majcenovic et al., however, demonstrated that the concentrations of both isotopically labeled DEDS and labeled CH_3CH_2SH decreased in wine during sixty days storage [30]. Further, a study by Ugliano et al. in 2012 showed that an increase in CH_3SH concentration did not lead to a corresponding decrease in DMS concentration in Shiraz wine [28]. Moreover, this hypothesis does not explain the increase in H_2S.

Another proposed mechanism for the formation of SLOs after bottling is that alkyl thiol esters are hydrolyzed during storage under acidic wine conditions. This was first suggested by Rauhut et al. in 1998 and shown to occur in certain wines [31]. Conversely, the same 2012 study by Ugliano et al. also found that a decrease in methyl thioacetate did not corresponding to an increase in CH_3SH in Shiraz wine. An additional hypothesis suggests that SLOs form in wine during storage due to the degradation of sulfur-containing amino acids. A recent study by Viviers et al. proposed this as a possible explanation for the increase of H_2S and CH_3SH in wine with

added reactive metals [32]. In fact, a 1971 study by Wainwright et al. showed that CH_3SH could develop in model beer spiked with methionine [33]. Later research by Pripis-Nicolau supports these findings: H_2S and CH_3SH could form from cysteine and methionine, respectively, in the presence of carbonyl compounds. While these studies demonstrate that amino-acid degradation could explain an increase in thiol concentrations under particular storage conditions, it likely requires the presence of some type of catalyst.

Lastly, H_2S could react with chemicals from exogenous sources to form more potent aroma compounds. This explanation has been reported in several food systems. For example, Patterson and Rhodes first described meat with "catty taint" in 1967. It was discovered that H_2S from the meat reacted with mesityl oxide in paint solvent to form the potent polyfunctional thiol, 4-MMP [34, 35]. In a more recent study by Mottram and Madruga, H_2S was shown to react with various alkanediones in an ethanol solution to form many volatile sulfur compounds [36]. In wine, Blanchard et al. demonstrated that furfural from oak staves and H_2S could react to form furfurylthiol during barrel fermentation [37]. Tominaga et al. also proposed that H_2S and benzaldehyde might react to form benzenemethanethiol found in Chardonnay, Sauvignon blanc, and Champagne [38, 39]. Until now, reactions between H_2S and chemical compounds present in flavoring agents have not been investigated as potential contributors to SLO formation in flavored wines.

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CHAPTER TWO

POST-BOTTLING DEVELOPMENT OF SULFUR-LIKE OFF AROMAS IN FLAVORED WINES

Abstract

The occasional appearance of low molecular weight thiols and sulfur-like off aromas (SLOs) in wines during anaerobic bottle storage is a well-recognized fault, but the appearance of this condition in flavored wines due to interactions between the wine and flavoring agent has not been previously reported. Sixteen flavoring agents were evaluated for their ability to form SLOs following their addition to wine and bottle storage. The flavors were spiked into a neutral wine, stored in a low-oxygen environment, and evaluated at one, three, and five months. After three months, wines spiked with one of the flavoring agents (clary sage) exhibited an SLO described as "skunky/burnt rubber." Although no H_2S was detected in the sample, the disappearance of the malodor following copper-fining indicated the compound was likely a thiol. GC-Olfactometry was used to identify 3-methyl-2-buten-1-thiol (3-MBT) as the compound responsible for the "skunky/burnt rubber" aroma. 3-methylbut-2-en-1-ol, a likely precursor of 3-MBT, was quantified by GC-TOF-MS and determined to be significantly higher in the clary sage flavor than in flavors that did not produce SLOs.

Introduction

Volatile sulfur-containing compounds are important contributors to wine aroma. While these compounds can contribute varietal character at certain concentrations, they are most often associated with off aromas. Examples of sulfur-containing aroma compounds found in wine include sulfides, disulfides, mercaptans, and thioacetates. Typical descriptors of these low-molecular weight compounds are cabbage, rotten egg, rubber, and onion [1, 2]. Furthermore, these compounds typically have low sensory thresholds, in the range of ng/L to µg/L, and therefore can impact wine-flavor even at very low concentrations [3, 4]. According to a recent study by Siebert et al., the sulfurous compounds that are most often observed in wines with SLOs in wine are H_2S and CH_3SH [4].

The emergence of SLOs in wine is caused by many complex chemical and biochemical pathways, only a few of which are well understood. Yeast produce volatile sulfur compounds, including H_2S and CH_3SH, during fermentation through amino acid metabolism. Concentrations in wine after fermentation are influenced by yeast strain, nutrient concentration, as well as fermentation and thermal stresses [3, 5]. It is also well known that yeast will directly reduce elemental sulfur present in grape must to H_2S, although this is only important if elemental sulfur-containing pesticides are applied late in the season [6]. While unpleasant, these sulfurous compounds can usually be remediated in the winery, e.g. by aeration or copper fining.

Of greater concern to winemakers is the formation of sulfur-like off aromas during anaerobic wine storage, particularly bottle storage. Several authors have demonstrated that anaerobic storage conditions can result in increased SLOs and/or concentrations of key volatile sulfur compounds in bottled wines. For example, research by Godden et al. investigated the influence of wine closure type on the sensory properties of Semillon wine. Wines bottled under

screw cap showed evidence of SLOs after evaluation at eighteen months [7]. Furthermore, a 2009 study by Lopes et al. showed that Sauvignon Blanc wines sealed under low-oxygen conditions had increased levels of H_2S after two years storage. Sensory analysis of the same wines indicated increased "rotten egg" and "putrefaction" aromas [8]. Ugliano et al. saw similar results in 2011: Sauvignon Blanc wines stored in a low-oxygen environment showed development of H_2S after six months [9]. This has also been seen in red wines. In a 2012 study by the same group, Shiraz wines stored under low-oxygen conditions showed increased concentrations of H_2S and CH_3SH [10]. A recent study by Viviers et al. investigated the influence of five metals and oxygen exposure on the concentration of sulfur-containing compounds in Shiraz and Chardonnay wines. Shiraz wines treated with copper evolved CH_3SH as dissolved oxygen levels decreased. The same phenomenon was seen with H_2S in Chardonnay wines [11].

The appearance and/or retention of SLOs during anaerobic storage are thus widely observed, but the mechanism for formation is still not well understood. An early hypothesis was that disulfides are converted to mercaptans under wine reducing conditions. Bobet et al. investigated the conversion of DEDS to CH_3CH_2SH in a wine model system at different pH levels. Predictive models from the study suggest that suprathreshold levels of CH_3CH_2SH could form in wine after seven hundred days of storage [12]. However, investigations with real wines do not support the disulfide precursor hypothesis. In fact, the 2012 study by Ugliano, et al. showed that an increase in CH_3SH concentration in stored Shiraz did not lead to a corresponding decrease in DMDS concentration [10]. The second mechanism proposed for the formation of SLOs during storage is that alkyl thiol esters are hydrolyzed to mercaptans during storage under acidic wine conditions. This was first suggested by Rauhut, et al. and shown to occur in certain

wines [13]. However, the same study by Ugliano, et al. also found that a decrease in methyl thioacetate did not correspond to an increase in CH_3SH [10]. Further, this mechanism does not explain H_2S increases during storage. Finally, the third SLO formation mechanism proposed is the degradation of sulfur-containing amino acids to form volatile thiols. Results from a study by Pripis-Nicolau, et al. indicated that H_2S could form from cysteine and CH_3SH from methionine in the presence of certain carbonyl compounds in a model wine system [14]. Further, results from the 2013 study by Viviers et al. suggests the release of H_2S and CH_3SH from amino acids could explain their increase during storage in the presence of reactive metals [11].

Another potential source of off-aromas is the reaction of wine components with exogenous compounds, such as those present in wine flavoring agents. Recent anecdotal evidence suggests that certain flavored wines can form SLOs when stored under low-oxygen conditions. Constellation Brands reported a fruit flavored wine that developed “burnt” or “skunky” aromas during bottle storage. The identity of the responsible odorants, the mechanism for their formation, and the prevalence of this phenomenon are not understood. As a result, it is not possible to identify potentially problematic flavoring agents and wine conditions that contribute to this fault prior to bottling. This research investigates the formation of SLOs in flavored wines during anaerobic storage. In the first part of the study, sixteen flavoring agents were spiked into white wine and inspected for their potential to form sulfur compounds during storage. One, which was part of the original formulation of the odorous Constellations wine, exhibited an odor described as “skunky” and “burnt.” The second part of the study focused on characterizing the compound contributing to this SLO and quantifying it in the odiferous samples, while the third part of the study investigated potential precursor compounds responsible for its formation.

Materials and Methods

Materials

Chemical Reagents and Standards. Potassium metabisulfite, sodium hydroxide, and copper sulfate were purchased from Fischer-Scientific (Hampton, NH). Ultra high purity grade compressed nitrogen was purchased from Airgas (Radnor, PA). 3-mercapto-2-butanone, 3-methyl-2-butenal, 3-methyl-2-buten-1-ol, sodium sulfate anhydrous, and thiourea were purchased from VWR International (Radnor, PA). (d_6)-3-Methyl-2-buten-1-ol was purchased from Santa Cruz Biotechnology, Inc (Dallas, TX). 3-methyl-1-butanethiol and sodium chloride was purchased from Sigma-Aldrich (St. Louis, MO). The 3-methyl-2-butene-1-thiol reference standard was synthesized according to protocol described by Holscher, et al. [15].

Flavoring Agents. Nat Strawberry Wonf 539371T, Mango Tommy Atkin NV46771, and Nat Mixed Berry Wonf 557511 C8 were supplied by Firmenich (Meyrin, Switzerland). Davana and Clary Sage were supplied by Synergy (Wauconda, IL). Marionberry Type Flavor 9152, Dual Berry 5330, Strawberry Type Flavor Nat 4131, and Blueberry Type Flavor Nat D100901 were supplied by Sethness Greenleaf (Chicago, IL). Pomegranate BN14290, Nat & Art Strawberry Type Flavor Artificial #29003, Nat & Art Strawberry Type Flavor Artificial #29880, Nat & Art Strawberry Type Flavor Artificial #29954, Nat Daiquiri Type Flavor 32072, and Natural Boysenberry Flavor Type 22582 were supplied by Ottens Flavors (Philadelphia, PA).

Wine. The neutral white wine used in this study was a 2011 Pinot Grigio delle Venezie from Castello di Gabbiano (Veneto, Italy) purchased at a local wine store (Ithaca, NY). The analytical parameters of the wine were as follows: pH was 3.4, residual sugar concentration was 1.5% w/v, titratable acidity was 7.3 g/L, ethanol concentration was 11.4% v/v, and free SO_2 was

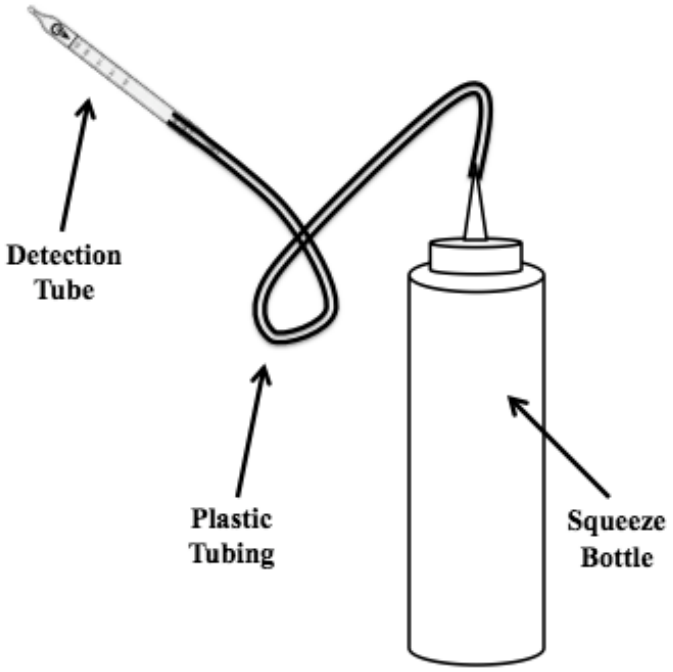
18.1 mg/L determined using Foss WineScan (Near IR), Foss FiaStar, and Gas Chromatography-FID by the Cornell University New York State Wine Analytical Laboratory.

Bottling studies to evaluate flavored wines for sulfur-like off aromas during storage

Wine-Flavor Sample Preparation. The free SO_2 of the white wine was adjusted to 50 mg/L with potassium metabisulfite. Sixteen flavoring agents were spiked individually into wine at 1% v/v. Wine-flavor samples were sparged with nitrogen gas for ten minutes and bottles were flushed with nitrogen gas prior to filling. Samples were then bottled without headspace in eleven 187 mL clear glass bottles (Waterloo Contain Company, Waterloo, NY) in an Atmosbag (Sigma-Aldrich, St. Louis, MO) filled with nitrogen gas. Bottles were sealed with oxygen barrier crown caps (LD Carlson Company, Kent, OH) using a hand bottle capper (Northern Brewer, Minneapolis, MN) inside of the Atmosbag. Wine-flavor samples were stored at room temperature with minimal light exposure. Control wine samples were bottled using the same protocol without added flavoring agents and stored under the same conditions.

Wine-Flavor Sample Evaluation. Samples were evaluated in duplicate at 1, 3, 5 months for production of SLOs and H_2S. Three people familiar with sulfur-containing compounds in wine performed initial sensory screen for SLOs. H_2S was quantified using Gastec 4LT gas detection tubes (Nextteq, Tampa, FL). The following method was adapted from Kwasniewski, et al [16]: a 30 g sample was added to squeeze bottle apparatus (Figure 1). Two Alka-Seltzer (Bayer, Leverkusen, Germany) tablets were added to apparatus and sealed tightly in order to sparge H_2S into attached detection tube. H_2S concentration was quantified by measuring color change in detection tube. The limit of detection is 1.1 ng/g for 30 g wine sample.

Figure 1. Squeeze bottle apparatus used to quantify free H_2S in wine-flavor samples after storage.



Exploration of potential contributors to sulfur-like off aroma in wine-flavor sample

Headspace SPME Analysis: Analyses of volatile compounds in the wine-flavor sample were carried out by measuring 10 mL of sample into 20 mL glass vials (22.5 X 95.5 mm, Supelco, Bellefonte, PA) with a PTFE stir bar (10 x 3 mm, VWR International, Radnor, PA). Extraction was performed using a 2 cm, 50/30 um, PVB/Carboxen/PDMS fiber (Supelco, Bellefonte, PA). The fiber was exposed to the headspace above the sample for 15 minutes while the sample was magnetically stirred. The fiber was then retracted and transferred to the injection port of the gas chromatograph.

GC-O Analysis: Analyses were performed using an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a flame ionization detector and sniff port (Datu, Inc., Geneva, NY). The injections were carried out in the splitless mode at 250°C. The

columns used were a DB-5 (30 m x 0.32 mm i.d., 0.25 um film thickness) from Agilent Technologies (Santa Clara, CA) and a ZB-WAX Plus (30 m x .53 mm i.d., 1 um film thickness) from Phemonenex (Torrance, CA). The GC method for the DB-5 column is as follows. The carrier gas was helium at a pressure of 10.1 psi. The oven temperature was programmed to rise from 35°C to 200°C at 4°C/min, then to 240°C at 30°C/min. The GC method for the ZB-WAX column is as follows. The carrier gas was helium at a pressure of 3.7 psi. The oven temperature was programmed to rise from 40°C to 230°C at 6°C/min. At the sniff port detector, the following parameters were selected: the temperature was set to 250°C, the airflow to 400 mL/min, the make-up flow to 45 mL/min, and the make-up gas was nitrogen. Sniffing time was approximately 28 minutes. Data was processed using Agilent ChemStation software.

Quantification of 3-methyl-2-buten-1-thiol formation in samples over time

Wine-Flavor Sample Preparation. Three clary sage flavors were selected for further bottling studies: Synergy Clary Sage Oil (Wauconda, IL), Plant Therapy Clary Sage Essential Oil (Twin Falls, ID), and Eden's Garden Clary Sage Therapeutic Grade Essential Oil (San Clemente, CA). The free SO_2 of the white wine was again adjusted to 50 mg/L with potassium metabisulfite prior to filling. Flavors were spiked into wine at .5% v/v. Samples were sparged with nitrogen for 15 minutes and bottled without headspace according to procedure described above. Control samples were also prepared using the same procedure: three clary sage flavors were spiked into model wine (pH 3, 12% ethanol v/v) and wine was bottled without any additional flavoring agents. Wine-flavor and control samples were stored at room temperature with minimal light exposure. This procedure was repeated once a month for three consecutive

months to produce three time points. In addition, wine-flavor samples were bottled one day prior to analysis to yield 'Month 0' time point.

Derivatization of 3-methyl-2-buten-1-thiol. 3-MBT was derivatized by adapting a protocol used for analysis of other volatile thiols in wines [17]. 40 mL of sample was added to a 60 mL clear glass screw cap vial (VWR International, Radnor, PA). Eighty µL of a 200 ppb 3-methyl-1-butanethiol in 2-propanol was spiked into the sample as an internal standard. The pH of the sample was then increased to 12 using 3M NaOH. Thirty µL of the derivatizing agent (100 µL of 100% pentafluorobenzyl bromide in 5 mL 2-propanol) and 9 mL of 1:3 v/v pentane:diethyl ether solution was added to the vial. The sample was agitated for 10 minutes at room temperature (Belly-Dancer, Stovall Life Science, Inc., Greensboro, NC). The organic layer was then separated from the aqueous layer and transferred to a 20 mL glass vial. The organic layer was dried down completely under nitrogen gas at room temperature. Finally, 10 mL of distilled water and 2 g sodium chloride were added to the glass vial.

Headspace SPME Analysis. HS-SPME analysis was completed using a Rail System (Leap Technologies, Naperville, IL). Extraction was performed using a 2 cm, 50/30 µm, PVB/Carboxen/PDMS fiber (Supelco, Bellefonte, PA). The sample was incubated for 15 minutes at 70°C while being agitated at 600 rpm. The compounds were then desorbed from the fiber into the GC injector in the splitless mode for 15 minutes at 250°C.

GC-TOF-MS Analysis. The quantification of the derivatized 3-MBT was carried out by gas chromatography time-of-flight mass spectrometry (GC-TOF-MS). Analyses were performed using an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA) coupled to a Pegasus 4D time-of flight mass spectrometer (LECO, St. Joseph, MI). The column used was a CP-Sil 8 (30 m x 0.25 mm i.d., 0.25 µm film thickness) from Agilent Technologies (Santa Clara,

CA). The carrier gas was helium at a target flow rate of 1.50 mL/min. The oven temperature was programmed to rise from 40°C to 100°C at 3°C/min, then to 240°C at 10°C/min. The mass spectrometry data was processed using LECO ChromaTOF software. The quantifying ion was *m/z:* 282 for 3-MBT (Figure 2) and *m/z:* 284 for the 3-methyl-1-butanethiol internal standard (Figure 3). The detection limit for this method was estimated by multiplying three times the noise area of the chromatogram for the lowest point on the calibration curve, and found to be on the level of low ng/L.

Figure 2. Mass spectra of 3-MBT-PFBn.

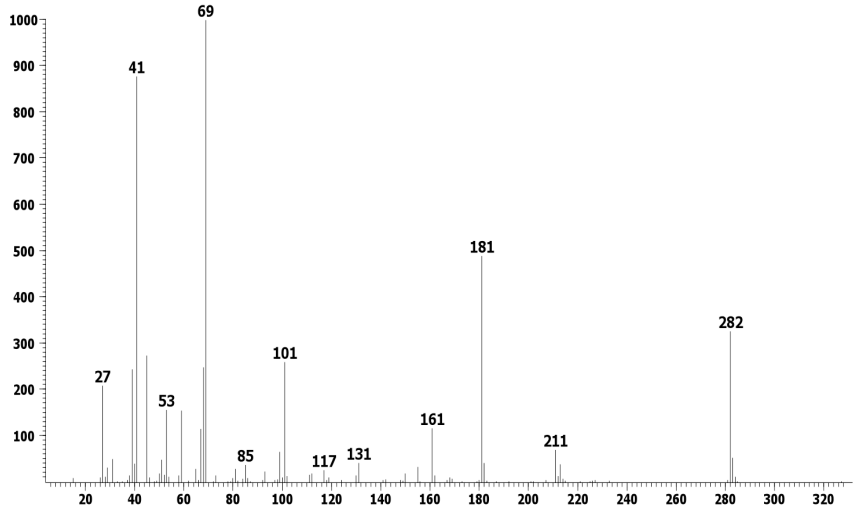
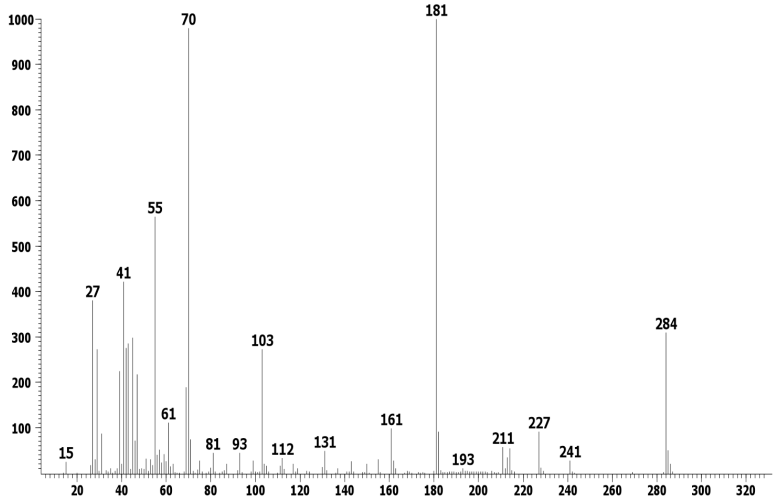


Figure 3. Mass spectra of 3-methyl-1-butanethiol-PFBn internal standard.



Identification and quantification of 3-methyl-2-buten-1-ol in flavors and wine

Sample Preparation. Model wine (pH 3, 12% ethanol v/v) was prepared according to protocol adapted from Danilewicz, 2007 [18]. The three clary sage flavors listed above and five additional flavors were selected for analyses (Table 3). Each flavor standard (0.5 mL) was spiked into 50 mL model wine to yield a final sample concentration of 1% v/v. Finally, 5 mL of sample, 5 mL of DI water, 3 g of sodium chloride, and 20 µL of d6-3-methyl-2-buten-1-ol as the internal standard were added to 20 mL glass vials.

Headspace SPME Analysis. HS-SPME analysis was again completed using the Rail System. Extraction was performed using a 2 cm, 50/30 µm, PVB/Carboxen/PDMS fiber. The sample was incubated for 10 minutes at 40°C while being agitated at 600 rpm. The compounds were then desorbed from the fiber into the GC injector in the splitless mode for 15 minutes at 250°C.

GC-TOF-MS Analysis. The quantification of 3-methyl-2-buten-1-ol was also carried out by gas chromatography time-of-flight mass spectrometry (GC-TOF-MS). Analyses were again performed using an Agilent 6890 gas chromatograph coupled to a Pegasus 4D time-of flight mass spectrometer. The column used was a CP-Sil 8 (30 m x 0.25 mm i.d., 0.25 µm film thickness) from Agilent Technologies (Santa Clara, CA). The carrier gas was helium at a target flow rate of 1.50 mL/min. The oven temperature was programmed to rise from 40°C to 100°C at 3°C/min, then to 240°C at 10°C/min. The mass spectrometry data was processed using LECO ChromaTOF software. The quantifying ion was *m/z:* 86 for 3-methyl-2-buten-1-ol and *m/z:* 92 for the deuterated internal standard (not shown).

Results and Discussion

Formation of sulfur-like off aroma in flavored wine during anaerobic bottle storage

Wine-flavor samples were inspected at 1, 3, 5 months for production of SLOs and H_2S. H_2S was not detected in concentrations above the 1.1 ng/g detection limit in any of sixteen flavored wine samples. Sensory results suggest that two of the sixteen flavored wine samples showed evidence of off-aroma formation. One flavoring agent (clary sage) developed "skunk/burnt rubber" aroma after 3 months. Table 1 below illustrates the sensory investigation results.

Table 1. Sensory data of wine-flavor and control samples at months 0, 1, 3, and 5.

Sample	Month 0	Month 1	Month 3	Month 5
Wine (Pinot Grigio)	No SLOs	No SLOs	No SLOs	No SLOs
Wine + Flavor (Davana) Sample	No SLOs	No SLOs	Off Aroma	Off Aroma
Flavor (Clary Sage) in Model Wine	No SLOs	No SLOs	No SLOs	No SLOs
Wine + Flavor (Clary Sage) Sample	No SLOs	No SLOs	Skunk, Burnt	Skunk, Burnt
14 Other Wine + Flavor Samples	No SLOs	No SLOs	No SLOs	No SLOs

Identification of 3-methyl-2-buten-1-thiol in wine-flavor sample by GC-O Analysis

Copper sulfate was added to the two wine-flavor samples that produced off aromas after three months in storage. The unidentified off aroma in the davana wine-flavor sample did not change, indicating that the compound responsible was unlikely to be a thiol. The "skunky" or "rubber-like" aroma in the clary sage wine-flavor sample seemed to dissipate upon copper addition; indicating that the compound might be a thiol.

GC-O identification of the SLO in the "skunky/rubber-like" clary sage wine-flavor sample was performed by comparing the odor and chromatographic retention index in both the DB-5 and ZB-WAX columns with those of pure or synthesized reference compounds injected

under the same conditions and/or reported in FlavorNet (Table 2). Comparison indicated that the odiferous compound is 3-methyl-2-buten-1-thiol.

Table 2. GC-O data from analyses of odorant standards and wine-flavor sample with SLO.

Compound/Sample	**DB-5 AVG RI**	**Wax AVG RI**	**Odor**
3-methylbutane-1-thiol	794	N/A	Skunk
3-mercapto-2-butanone	804*	1152*	Onion
Wine-Flavor Sample	*816*	*1116*	*Skunk, rubber*
3-methyl-2-buten-1-thiol	817	1117	Amine, smoke, skunk*

*Retention times and/or odor descriptor from FlavorNet.

3-MBT is a well-known contributor to the skunk aroma in light-struck beer [19]. It is also a key aroma compound in freshly roasted coffee and was recently found in virgin olive oil [15, 20-22]. 3-MBT has also been found in wine at low concentrations in a rare Spanish varietal [23] and in Sauternes [24].

Quantification of 3-methyl-2-buten-1-thiol formation in samples over time

To measure 3-MBT in the wine-flavor samples, the compound was derivatized by reacting pentafluorobenzyl bromide with the thiol group. This protocol allows for increased sensitivity and selectivity in the analysis of most polyfunctional thiols. Recent work suggests that detection limits between .9 – 17 ng/L can be achieved for certain sulfur-conjugated thiols in wine using conventional GC-EI-MS [17]. The detection limit in this study is similar, it was estimated to be at the level of low ng/L. Still, 3-MBT was not detected in any of the wine-flavor samples in concentrations above this detection limit. It is possible this is due to matrix effects from the essential oils in the flavoring agents, which are expected to decrease analyte volatility and compete for binding sites on the SPME fiber. We observed a 20-fold decrease the signal of the

internal standard in real wine-flavor samples vs. in the calibrated samples in model wine, although further research is required to verify this hypothesis.

Proposed mechanisms for 3-MBT formation in wine and beer

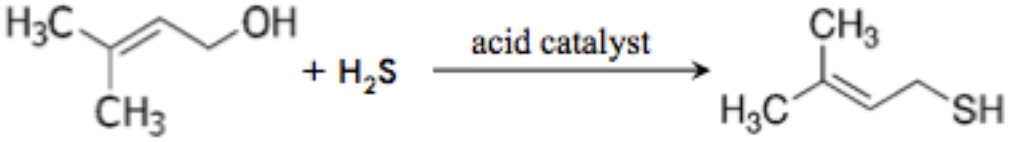
There are several proposed formation mechanisms for 3-MBT in beverages. The most common example occurs in light-struck beer when iso-alpha acids in hops are photodegraded into free radicals that then react with a thiol donor to create 3-methyl-2-buten-1-thiol. This was first proven by Gunst, et al. in 1978 and details have been confirmed in many recent studies [19, 25, 26].

Similar to 4-mercapto-4-methyl-pentan-2-one and 3-mercapto-1-hexanol in Sauvignon Blanc, it is possible that 3-MBT can be released from a sulfur-conjugated precursor. In a recent study by Gros, et al. it was found that a sulfur-conjugate of 3-MBT exists in particular hop varieties. The compound can then be enzymatically released by *β*-lyase from yeast during fermentation [27]. This has only been shown in model solutions. The S-conjugated precursor has not been found in plant essential oils and perhaps more importantly, *β*-lyase activity only occurs during fermentation, which does not apply to stored samples. Therefore, this explanation is unlikely to be responsible for 3-MBT formation in the wine-flavor samples in this study.

Finally, it has been shown that 3-MBT can be formed through a nucleophilic substitution reaction between 3-methyl-2-buten-1-ol and H_2S, where the hydroxyl group of the alcohol is substituted for a thiol group. Gros, et al. demonstrated this mechanism occurs in model beer media by spiking wort with 3-methyl-2-buten-1-ol and quantifying the thiol after fermentation [28]. This reaction also takes place under the pyrolytic conditions of coffee roasting [15]. In wine, a study by Bailly, et al. proposed that this pathway is responsible for 3-MBT formation in

Sauternes and that it is catalyzed by low pH conditions [24]. This mechanism seems the most likely explanation for 3-MBT formation in the wine-flavor sample in this research (Figure 4).

Figure 4. Hypothetical reaction between H_2S and 3-methylbut-2-en-1-ol to form 3-MBT.



Identification and quantification of 3-methyl-2-buten-1-ol in flavors and wine

To investigate the role of 3-methylbut-2-en-1-ol as a precursor for 3-MBT, its concentration was measured in three different clary sage flavoring agents and five flavoring agents that did not form SLOs during storage with wine. While the Pinot Grigio wine alone contains 3-methylbut-2-en-1-ol at low concentrations, the three clary sage flavoring agents contain the potential precursor at significant concentrations, between 150 – 450 µg/L (Table 3, Figure 5). 3-methylbut-2-en-1-ol was not detected in the flavoring agents that did not form SLOs (Figure 6). This suggests that 3-methylbut-2-en-1-ol is a likely precursor to 3-MBT in the wine-flavor samples in this study.

In order to form 3-MBT, 3-methyl-2-buten-1-ol from the flavoring agents must react with H_2S present in the wine, which could either develop during storage [4, 8, 9] or exist at subthreshold concentrations prior to bottling. Because the detection threshold in wine for 3-MBT (.7 ng/L) is much lower than the threshold for H_2S (1.1 µg/L), H_2S present at high enough levels to form 3-MBT at suprathreshold levels may not even be detected in the wine.

Table 3. Concentrations of 3-methyl-2-buten-1-ol in eight flavoring agents and Pinot Grigio control wine without added flavor in 187 mL samples.

Flavor Sample	Concentration in 187 mL Sample (ug/L)
Pinot Grigio (no flavor spike)	16.2
Clary Sage (Plant Therapy)	314.4
Clary Sage (Eden's Garden)	207.6
Clary Sage (Synergy)	877.1
Nat Strawberry (Firmenich)	n.d.
Marionberry (Synergy)	n.d.
Blueberry (Sethness Greenleaf)	n.d.
Boysenberry (Ottens)	n.d.
Pina Colada (Givaudan)	n.d.

Figure 5. Gas chromatogram showing deuterated 3-methylbut-2-en-1-ol internal standard and 3-methylbut-2-en-1-ol peak in Synergy clary sage flavoring agent.

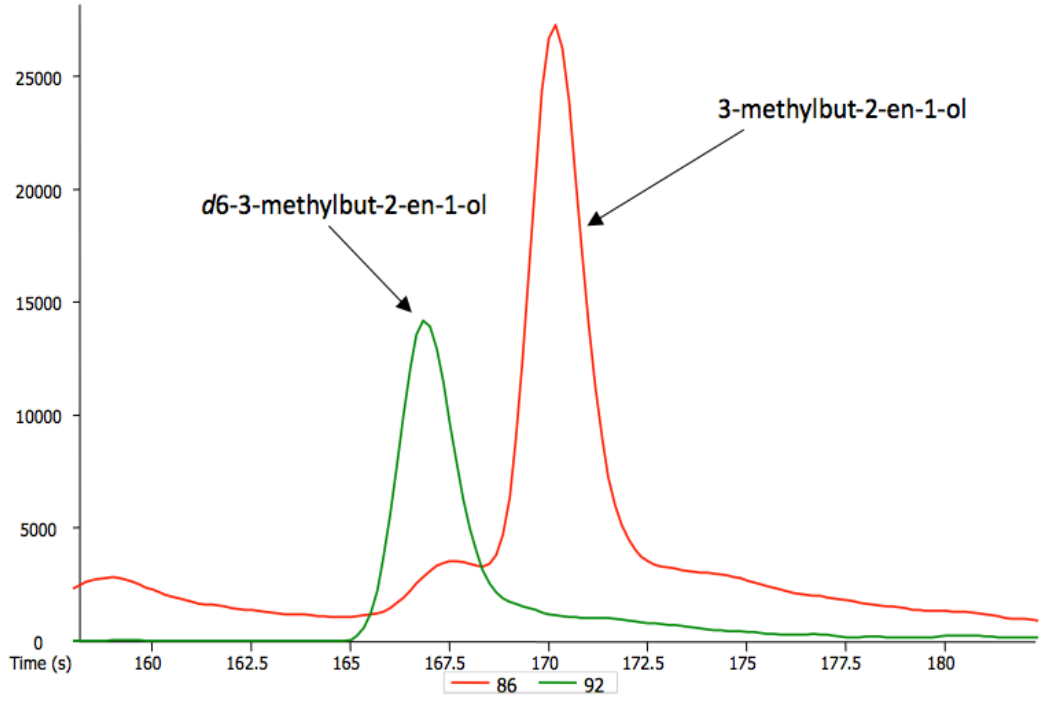
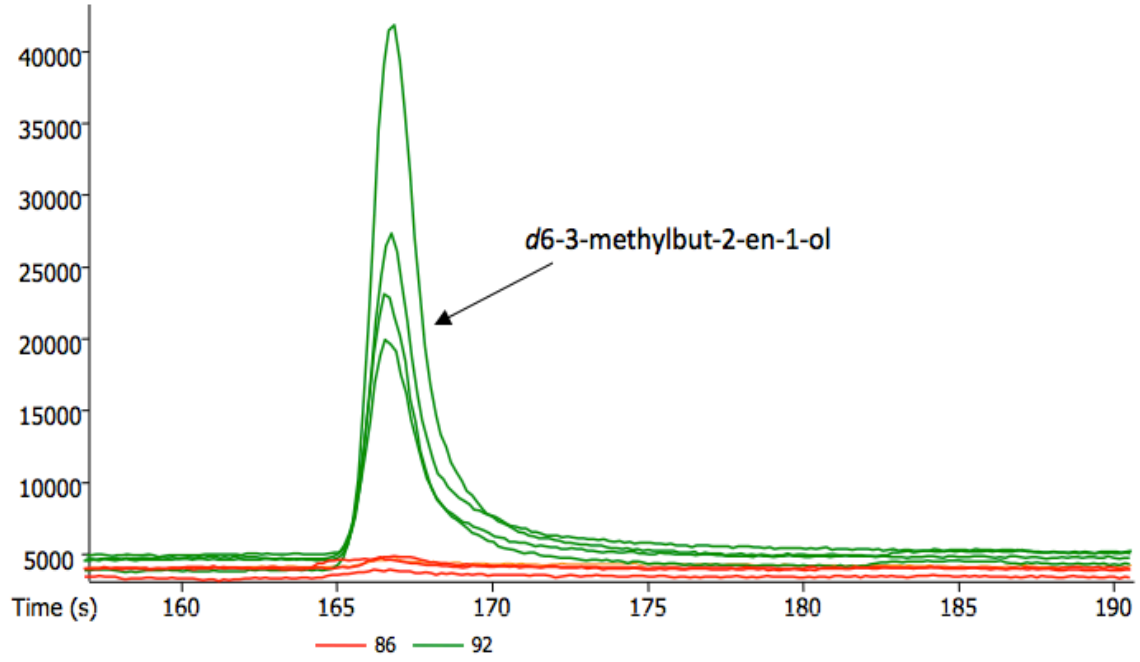


Figure 6. Gas chromatogram showing deuterated 3-methylbut-2-en-1-ol internal standard in non- SLO producing flavoring agents.



Conclusion

This study found that 3-methyl-2-buten-1-thiol is an important contributor to sulfur-like off aromas that develop post-bottling in flavored wines. While it is well known that this compound is responsible for the "skunk" aroma in light-struck beer, it has rarely been found in wine. This is the first report of 3-MBT in wines with added flavoring agents. We hypothesize that 3-MBT forms by the reaction of 3-methylbut-2-en-1-ol present in flavoring agents with H_2S present in wine, which is catalyzed by acidic conditions.

In order to prevent this fault from occurring in the future, several prevention strategies could be employed. First, flavoring agents could be screened for precursors (e.g. 3-methylbut-2-en-1-ol) prior to use. Second, flavoring agents could be spiked into a model wine with high H_2S concentration and evaluated for off aroma after storage. Third, H_2S concentration in wine could be decreased prior to bottling.

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